

Agathisflavone Enhances Retinoic Acid-Induced Neurogenesis and Its Receptors α and β in Pluripotent Stem Cells

Bruna S. Paulsen,^{1,*} Cleide S. Souza,^{1,2,*} Leonardo Chicaybam,³ Martin Hernán Bonamino,³ Marcus Bahia,⁴ Sílvia Lima Costa,² Helena L. Borges,¹ and Stevens K. Rehen¹

Flavonoids have key functions in the regulation of multiple cellular processes; however, their effects have been poorly examined in pluripotent stem cells. Here, we tested the hypothesis that neurogenesis induced by *all-trans* retinoic acid (RA) is enhanced by agathisflavone (FAB, *Caesalpinia pyramidalis* Tull). Mouse embryonic stem (mES) cells and induced pluripotent stem (miPS) cells growing as embryoid bodies (EBs) for 4 days were treated with FAB (60 μ M) and/or RA (2 μ M) for additional 4 days. FAB did not interfere with the EB mitotic rate of mES cells, as evidenced by similar percentages of mitotic figures labeled by phospho-histone H3 in control (3.4% \pm 0.4%) and FAB-treated groups (3.5% \pm 1.1%). Nevertheless, the biflavonoid reduced cell death in both control and RA-treated EBs from mES cells by almost 2-fold compared with untreated EBs. FAB was unable, by itself, to induce neuronal differentiation in EBs after 4 days of treatment. On the other hand, FAB enhanced neuronal differentiation induced by RA in both EBs of mES and miPS. FAB increased the percentage of nestin-labeled cells by 2.7-fold (mES) and 2.4 (miPS) and β -tubulin III-positive cells by 2-fold (mES) and 2.7 (miPS) in comparison to RA-treated EBs only. FAB increased the expression of RA receptors α and β in mES EBs, suggesting that the availability of RA receptors is limiting RA-induced neurogenesis in pluripotent stem cells. This is the first report to describe that naturally occurring biflavonoids regulate apoptosis and neuronal differentiation in pluripotent stem cells.

Introduction

THE CANONICAL METHOD to induce neuronal differentiation in pluripotent stem cells, such as embryonic and induced pluripotent stem (iPS) cells, requires retinoic acid (RA), which downregulates the expression of mesodermal genes and upregulates the expression of neural genes, such as nestin, β -tubulin, NCAM, PAX, GAD 67, GAD 65, and neurofilament L [1]. Nevertheless, the apoptotic genes caspase 3 and caspase 6 are also induced by RA [2], which reduces the effectiveness of RA as a neural morphogen in vitro.

Flavonoids are natural polyphenolic compounds present in a variety of vegetables of human intake [3]. These compounds showed several biological properties, including effects on cell differentiation [4,5]. For example, fisetin, luteolin, quercetin, and isorhamnetin induce neural differentiation in pheochromocytoma PC12 cells [6]; icariin and icaritin induce cardiac differentiation in embryonic stem (ES) cells [7,8], whereas biflavonoids such as bilobetin, sciadopitysin, and 7,4',7'',4'''-O-methyl-amentoflavone induce the differentiation of osteoblasts [9].

Flavonoids are also able to reduce cell death [4,5]. Apigenin and kaempferol protect rat cortical neurons against amyloid- β -induced neurotoxicity by inhibiting caspase 2, 3, 8, and 9 [10], whereas the polyphenol epigallocatechin gallate inhibits the fibrillogenesis of both α -synuclein and amyloid- β [11], associated with Alzheimer's and Parkinson's diseases, respectively.

Here, we investigated the biological effects of agathisflavone (FAB; Supplementary Fig. S1E; Supplementary Data are available online at www.liebertonline.com/scd), a biflavonoid extracted from *Caesalpinia pyramidalis* Tull, an abundant plant at the northeast of Brazil, in mouse ES (mES) and miPS cells.

Materials and Methods

Agathisflavone

Agathisflavone (FAB) (Supplementary Fig. S1E) was extracted from *C. pyramidalis* Tull leaves as previously described [12], stored at 50 mM in dimethyl sulfoxide

¹Instituto de Ciências Biomédicas, Laboratório Nacional de Células-Tronco Embrionárias, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil.

²Instituto de Ciências da Saúde, Universidade Federal da Bahia, Salvador, Brazil.

³Divisão de Medicina Experimental, Instituto Nacional do Câncer, Rio de Janeiro, Brazil.

⁴Universidade Federal do Recôncavo da Bahia, Recôncavo, Brazil.

*These authors equally contributed to this work.

(DMSO; Sigma Chemical Co), and kept out of light at 4°C until use.

Cell culture

mES cells, named as USP1, were kindly provided by Dr. Lygia Pereira (University of São Paulo-USP) and Dr. Irina Kerkis (Federal University of São Paulo-Unifesp). mES cells and miPS cells were grown in ES medium as previously described [13,14]. Briefly, cell colonies were grown on mouse embryonic fibroblast (MEF), mitotically inactivated with mitomycin C (Sigma), in 15% knockout serum replacement (KSR; Gibco)–DMEM/F12 (Gibco) supplemented with 1% nonessential amino acid solution (Gibco), 2 mM L-glutamine (Gibco), 0.1 mM β -mercaptoethanol (Gibco), 40 μ g/mL gentamicin sulfate (Scheing-Plough), and 0.2% of conditioned medium of CHO (Chinese hamster ovary) cells producing leukemia inhibitory factor (LIF).

For immunocytochemistry, mES or miPS colonies were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min. The expression of octamer-binding transcription factor 4 (Oct4), a transcriptional factor of pluripotent stem cells, was detected by immunofluorescence assay. Oct4 was uniformly expressed among mES colonies, but not detected on isolated nuclei corresponding to MEF cells (Supplementary Fig. S1B, C). As expected, western blot analysis showed that Oct4 was only detected in mES cells and not on MEFs (Supplementary Fig. S1D).

Retrovirus production

The human complementary DNAs of Oct4, sex-determining region Y-box 2 (SOX2), Krueppel-like factor 4 (Klf4), and cellular-myelocytomatosis viral oncogene homolog (c-Myc) inserted into moloney-based retroviral vectors (pMXs) were obtained from Addgene. Human embryonic kidney 293T cells were utilized as packaging cells to the virus production after calcium phosphate-based transfection with pMXs (20 μ g), pMDM (encoding mlv GAG/POL) (10 μ g), and pMD.G (encoding VSV G) plasmid (6 μ g). The viral supernatants were collected at 48 and 72 h posttransfection, filtered (0.22 μ m), concentrated at 32,000 g for 60 min, and incubated in a 2-round transduction on MEF (see below and Supplementary Fig. S2A).

Generation of iPS cells

For the generation of iPS cells, MEFs (Supplementary Fig. S2B) were transduced with concentrated supernatants of 4 retroviruses containing Oct4, SOX2, Klf4, and c-Myc. To improve the transduction, Polybrene (Sigma) was used at 8 μ g/mL and the plate was centrifuged at 540 g for 45 min. Two rounds of transduction were performed, with a recovery period of 24 h (d-2 and d-1; see Supplementary Fig. S2A). The medium was changed at day 0, and additional 48 h of recovery time was allowed in 10% fetal bovine serum (FBS; Gibco)–DMEM/F12 (Gibco) supplemented with 2 mM L-glutamine (Gibco), 0.1 mM β -mercaptoethanol (Gibco), and 40 μ g/mL gentamicin sulfate (Scheing-Plough). At d2, cells were cultured in ES cell medium (KSR+LIF), as described above, and treatment with the histone deacetylase inhibitor, valproic acid (VPA; 1 mM), which has been described to enhance the efficiency of iPS colonies formation [15], was

started and lasted a week. At d3, 2.5×10^5 cells were plated on 2% gelatin-coated 35-mm plate with feeder layer (MEF cells). At 15 days posttransduction, miPS colonies were selected based on ES cell-like colony morphology. The selected colonies were expanded and maintained at the same conditions as mES cells (see Supplementary Fig. S2A). VPA (Sigma) stock solutions were made in PBS.

Herein, our experiments were performed with an miPS clone called i-Rio, which is very similar morphologically to mES cells (Supplementary Fig. S2C). Our miPS cells were positive for the following pluripotency markers: Oct4 (Supplementary Fig. S2D, I), SOX2 (Supplementary Fig. S2F), SSEA-1 (Supplementary Fig. S2G), and phosphatase alkaline (data not shown). As expected, they were able to form embryoid bodies (EBs) (Supplementary Fig. S2J), and as a source to further confirm pluripotency, when replated onto gelatin, these EBs were capable to spread out cells from the 3 germ layers: endoderm (alpha fetoprotein; Supplementary Fig. S2K), mesoderm (smooth muscle actin; Supplementary Fig. S2L), and ectoderm (nestin; Supplementary Fig. S2M).

Formation and treatment of EBs

On the first day of culture, day 0, mES and miPS cells (3×10^6) were passed to a 6-well plate covered with 2% gelatin (Sigma) and cultured for 48 h (d0–d2) in 15% KSR-DMEM/F12 supplemented as above (ES medium) (Supplementary Fig. S1A). At day 2, colonies were treated with TrypLE™ Express (Invitrogen) for 5 min at 37°C, transferred to non-adherent plate dishes with DMEM/F12 medium with 15% FBS (Gibco) and 1% of conditioned medium of CHO cells producing LIF, and cultured for 4 days (from d2 to d6). Spherical cell aggregates (EBs) were formed between d2 and d6 in DMEM/F12 with 15% FBS. EB treatments were performed from d6 to d10 in LIF-free medium and 15% FBS. RA (Sigma) was used to induce neuronal differentiation. At d6, EBs were divided in 4 groups: (a) 0.1% vehicle (DMSO); (b) 2 μ M RA; (c) 60 μ M FAB; (d) 2 μ M RA + 60 μ M FAB. The medium was changed every other day. At day 10 (d10), EBs were fixed in 4% paraformaldehyde and processed for obtaining 10 μ m of cryosections as previously described [16]. In all experiments, 0.1% vehicle (DMSO) group was determined as control.

To allow neuronal migration in some experiments, at d10, EBs were plated onto 1 μ g/mL laminin (Invitrogen) and 1 μ g/mL fibronectin (Invitrogen)-coated dishes and cultured in basal medium DMEM/F12 with N2 supplement and 20 ng/mL of fibroblast growth factor-2 for additional 4 days [13].

Immunofluorescence analyses

Prior immunofluorescence assay slides with EB frozen sections were heated in a boiling bath of citrate buffer (0.01 M, pH 6.0) for 10 min. Colonies were treated with 0.3% of Triton X-100/PBS (Isosfar), whereas EBs were treated with 0.5% Triton X-100 in 50 mM NH_4Cl /PBS for 5 min. After incubation with 5% bovine serum albumin (Invitrogen) for 30 min, samples were incubated with the primary antibody overnight. After washing, cells were incubated for at least 1 hour with the secondary antibodies: goat anti-mouse IgG conjugated to Alexa Fluor 546 (Molecular Probe; 1:400) or goat anti-rabbit IgG conjugated to Alexa Fluor 488 (Molecular Probe;

1:400). The samples were washed with PBS and nuclear staining was performed with 1.0 µg/mL solution of 4',6-diamidino-2-phenylindole (DAPI) [13]. Images of cells were accessed with optic fluorescence microscopy (Nikon T300).

Antibodies used to assess pluripotency were anti-OCT 3/4 (Santa Cruz Biotechnology; mouse, 1:100), anti-SOX2 (Chemicon; rabbit, 1:100), and anti-SSEA1 (Chemicon; mouse, 1:100). The 3 germ layers were detected with endoderm-anti-alpha fetoprotein (Santa Cruz Biotechnology; rabbit, 1:100), mesoderm-anti-smooth muscle actin (Chemicon; mouse, 1:100), and ectoderm-anti-nestin (Chemicon; mouse, 1:100). To assess cellular mitosis rate, histological slices of EBs were incubated with polyclonal antibody anti-phospho-histone H3 (Upstate; rabbit, 1:400). Antibody anti-nestin (Chemicon; mouse, 1:100) and anti-β-tubulin III (Covance and Sigma; mouse, 1:100) were used to label, respectively, neural progenitor cells and neuronal cells in EB slices. Antibodies used to verify the expression of both RA receptors were anti-RARα and anti-RARβ (Santa Cruz Biotechnology; rabbit, 1:100).

For analysis of apoptosis in EBs, DNA fragmentation was detected by the terminal-deoxynucleotidyl-transferase dUTP nick-end labeling (TUNEL; Promega) on frozen EB sections, performed according to the manufacturer's protocol [13,17]. Cell death was also identified by cleaved caspase-3 immunofluorescence analysis using the antibody anti-cleaved caspase-3 (Cell Signalling; rabbit, 1:100).

Alkaline phosphatase staining

Alkaline phosphatase (a stem cell membrane marker associated with undifferentiated pluripotent stem cell) staining was performed in miPS colonies with the Alkaline Phosphatase Detection Kit (Millipore) to further detect pluripotency of these cells.

Quantitative analysis

The percentages of positive cells labeled with nestin, β-tubulin III, TUNEL, phospho-histone H3, or cleaved caspase-3 were calculated as follows: at each EB section, positive cells were counted, as well as the total number of cells stained with DAPI. For quantification, photomicrographs taken under 400× microscope magnification were 150% amplified on Adobe Photoshop for visualization of individual cells within the EB section. A multiplatform software [Embryonic Stem Cell Counter (ESCC)] was used for counting DAPI-positive cells from photomicrographs of EB sections. The software can be freely downloaded (<http://escs.inf.puc-rio.br/>) and is detailed in www.ic.uff.br/iwssip2010/Proceedings/nav/papers/paper_93.pdf. An average of 30 EBs (~12,000 DAPI-positive cells) were counted per group and each graphic expresses the mean of the average percentages of at least 3 independent experiments. Statistical analysis was performed using 1-way analysis of variance followed by Newman-Keuls posttest.

Semiquantitative analysis of the immunofluorescence of RAR alpha and beta were carried out as follows: photomicrographs were taken with the same fluorescence intensity under confocal microscope (Leica TCS SP5 AOBS). Once converted to grayscale, Image J 1.4.3U software was used to measure the area and to calculate the fluorescence intensity of each EB. The fluorescence intensity of EBs was subtracted

from the background. Graphics represent the rate of fluorescence intensity per EB area. We counted about 20 EBs per group and each graphic expresses the mean of the average rates of at least 3 independent experiments. Statistical analysis was performed using unpaired Student's *t*-test.

Results

Agathisflavone enhances neuronal differentiation induced by RA in EBs

To test the effects of FAB on ES neuronal differentiation, EBs were treated with RA (2 µM), FAB (60 µM), or both together (RA 2 µM and FAB 60 µM) (Supplementary Fig. S1A, F). On day 10, the percentage of neural progenitors within the aggregates was determined (Fig. 1A, B). No differences in the number of nestin-positive cells were observed between control (2.1%±0.7%) and FAB-treated cells (0.4%±0.3%); however, when FAB was combined with RA (RA+FAB group), the percentage of neural progenitor cells increased by 2.7 times (9.6%±0.6% in RA alone and 26%±6.5% in the RA+FAB) (Fig. 1A, B).

To confirm the number of young neurons, we assessed the percentage of β-tubulin III-positive cells, which were generated after RA or RA+FAB treatment. RA increased the number of β-tubulin III-positive cells (12%±2.1%; Fig. 2A, B) in comparison to controls (2.2%±0.3%; Fig. 2A, B), whereas FAB alone did not increase the number of β-tubulin III-positive cells (1.8%±0.9%). However, FAB together with RA (RA+FAB) doubled the percentage of β-tubulin III-positive cells (24.2%±4.3%) in relation to RA alone (12%±2.1%; Fig. 2A, B). To better compare the amount of neuronal cells between the treatments, EBs were plated on laminin/fibronectin-coated plates and cultured for additional 4 days in neurobasal medium free of RA or FAB. A subset of migrating β-tubulin III-positive cells with long processes emerged from EBs (Fig. 2C). Yet, the number of β-tubulin III-labeled cells migrating out of EBs after treatment with the combination of RA+FAB was visibly higher than in EBs treated with RA only (Fig. 2C). These results suggest that FAB enhances RA-induced neural differentiation in ES cells.

Agathisflavone also enhances neural differentiation triggered by RA in iPS cells

Colonies of miPS (Supplementary Fig. S2C) were generated from MEF cells by reprogramming. Pluripotency was confirmed by immunofluorescence for specific markers of undifferentiated ES cells (OCT-4, SOX2, and SSEA1; Supplementary Fig. S2D-I) and also their ability to originate cells from ectoderm, mesoderm, and endoderm (Supplementary Fig. S2J-M). Administration of FAB on miPS EBs did not increase the percentage of cells labeled for nestin (4.9%±3.0%) or β-tubulin III (0.8%±0.4%) in comparison to the percentage of nestin (1.9%±0.2%) or β-tubulin III (5.2%±2.5%)-labeled cells in control EBs (Figs. 3 and 4). As we observed with mES EBs, FAB together with RA increased the percentage of nestin-labeled cells by 2.4 times (46.3%±1.5% RA+FAB) in comparison to the percentage nestin-positive cells in RA only (19.3%±4.8%) (Fig. 3). The amount of β-tubulin III-positive cells was also increased in 2.7-fold by FAB+RA (36.9%±11.3%) when compared to RA treatment alone (13.9%±3.1%)

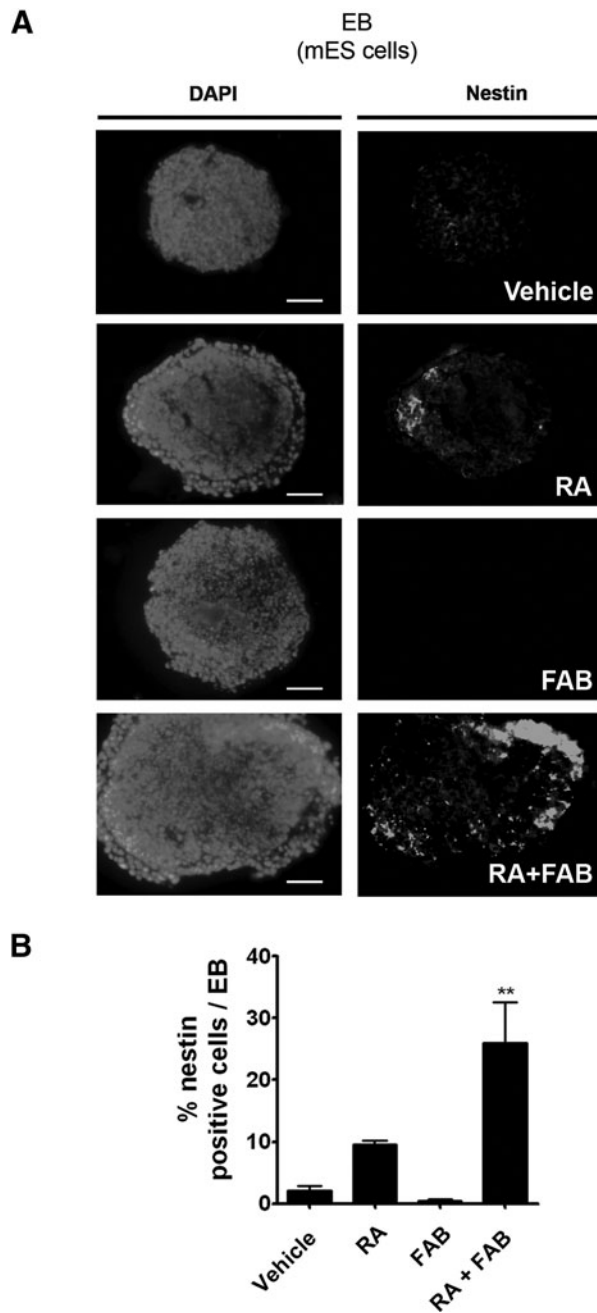


FIG. 1. Agathisflavone increases the number of neural progenitor cells induced by RA in mES cells. **(A)** EBs of USP1 mES cells treated for 4 days with 0.1% of vehicle (DMSO) as control or treated with 2 μ M RA, 60 μ M agathisflavone (FAB), or the combination of 2 μ M RA plus 60 μ M FAB (RA+FAB) were immunostained for anti-nestin; nuclei were counterstained with DAPI. **(B)** Quantification of the percentage of nestin-positive cells per EB (average of 30 EBs counted per group) with the indicated treatments. The results are the mean \pm SE of 3 independent experiments. A statistically significant difference was detected when RA+FAB was compared with the RA group (** P < 0.01, 1-way ANOVA), although the significance was higher between the RA+FAB and either the vehicle or FAB group (** P < 0.001). Scale bars correspond to 50 μ m. EBs, embryoid bodies; mES, mouse embryonic stem cells; DMSO, dimethyl sulfoxide; RA, retinoic acid; DAPI, 4',6-diamidino-2-phenylindole; SE, standard error; ANOVA, analysis of variance.

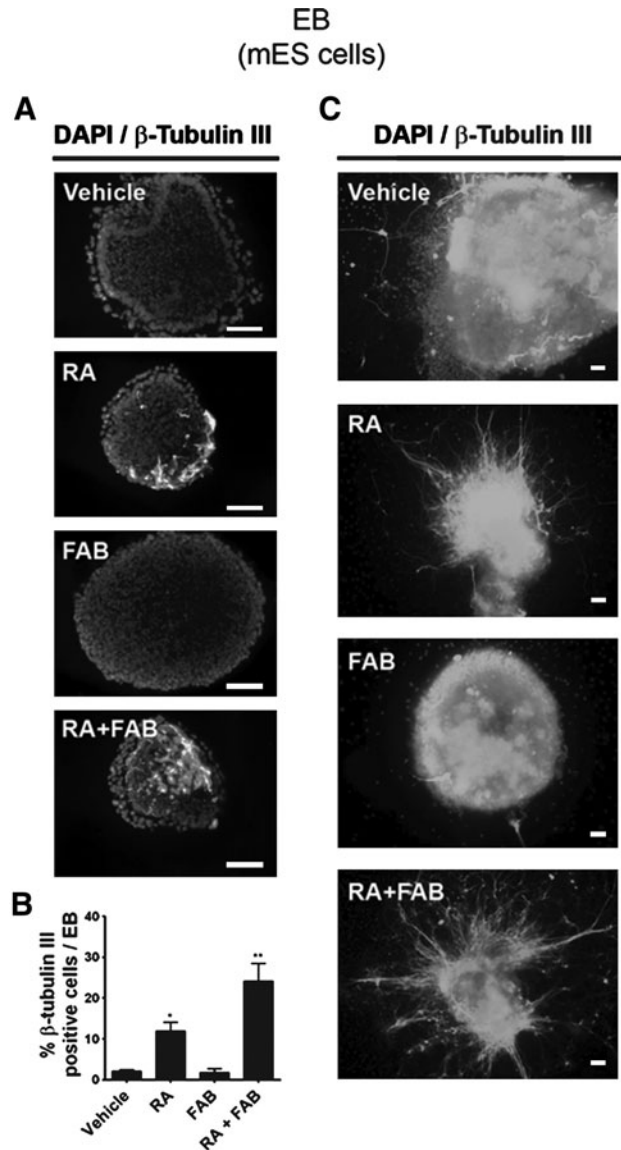


FIG. 2. Agathisflavone increases the number of young neurons induced by RA in mES cells. **(A)** Photomicrographs of frozen sections of EBs immunostained for anti- β -tubulin III and counterstained with DAPI. EBs derived from mES cells were incubated with 0.1% of vehicle (DMSO), 2 μ M RA, 60 μ M agathisflavone (FAB), or the combination of 2 μ M RA plus 60 μ M FAB (RA+FAB) for 4 days. **(B)** Quantification of the percentage of β -tubulin III-positive cells per EB as indicated treatments. The results are the mean \pm SE; n = 3. ** P < 0.01 when RA+FAB was compared with the RA group. RA differs from either vehicle or FAB with * P < 0.05 and from RA+FAB with ** P < 0.001 (1-way ANOVA). **(C)** EBs after 4 days of treatment were plated onto fibronectin/laminin-coated dishes in control medium for additional 4 days. Photomicrographs show that the amount of β -tubulin III-labeled tangles migrated out of the EBs periphery in the RA+FAB group was visibly greater when compared with the RA group. Scale bar corresponds to 50 μ m.

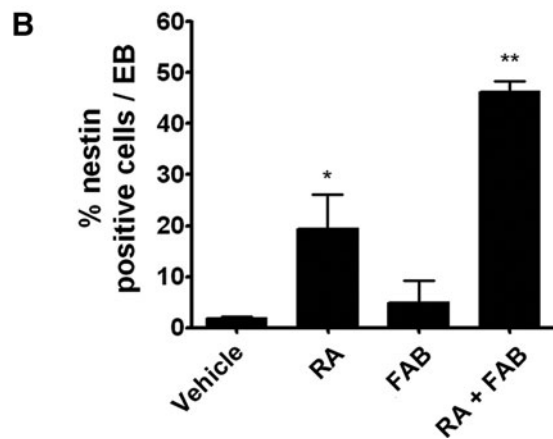
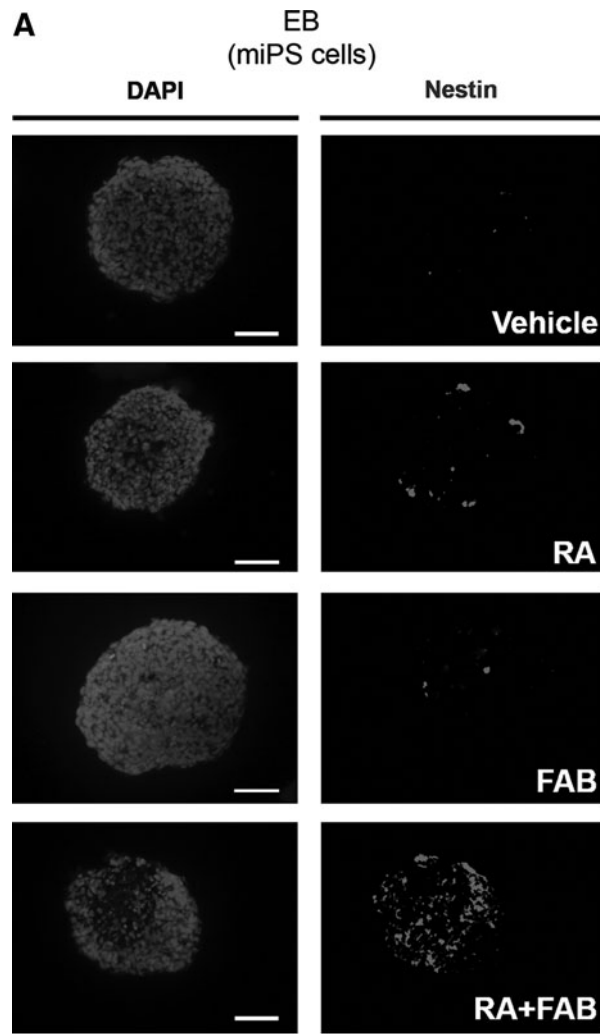


FIG. 3. Agathisflavone increases the number of neural progenitor cells induced by RA in miPS cells. **(A)** EBs derived from miPS cells and submitted to incubation with 0.1% of vehicle (DMSO), 2 μ M RA, 60 μ M agathisflavone (FAB), or the combination of 2 μ M RA plus 60 μ M FAB (RA+FAB) were immunostained for anti-nestin and nuclei were stained with DAPI. **(B)** Quantification of the percentage of nestin-positive cells per EB; the results are the mean \pm SE; $n=2$. ** $P < 0.01$ when RA+FAB was compared with RA; RA differs from vehicle or FAB with * $P < 0.05$. Scale bar corresponds to 50 μ m. miPS, mouse induced pluripotent stem cells.

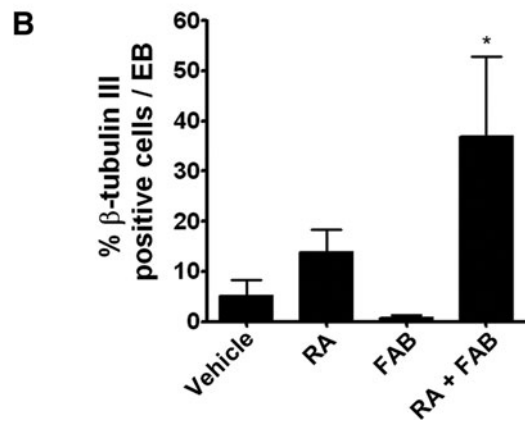
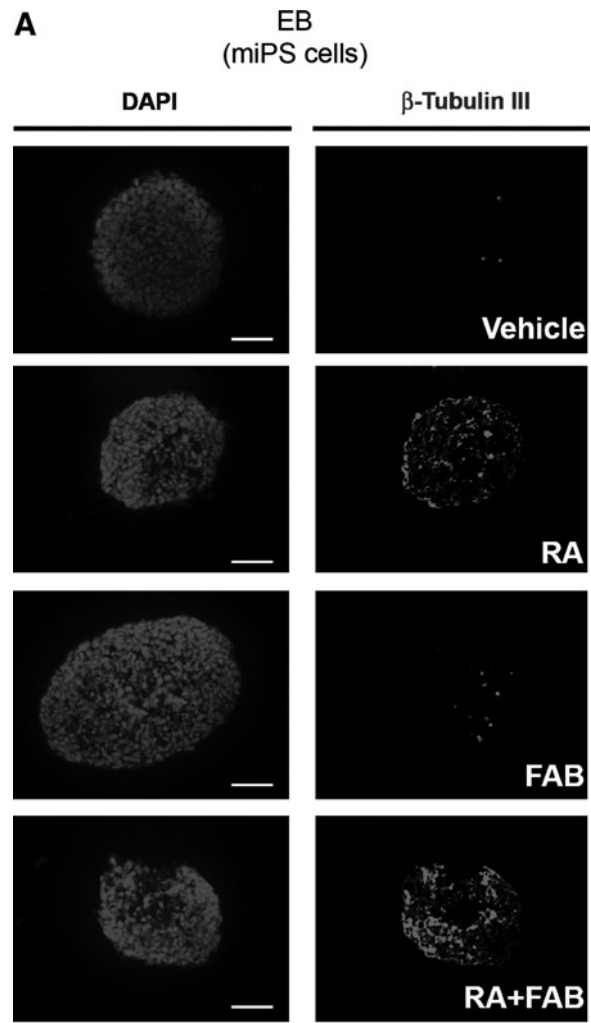


FIG. 4. Agathisflavone increases the number of young neurons induced by RA in mouse iPS cells. **(A)** Photomicrographs of frozen sections of EBs immunostained for anti- β -tubulin III; nuclei were counterstained with DAPI. EBs derived from miPS were incubated with 0.1% of vehicle (DMSO), 2 μ M RA, 60 μ M agathisflavone (FAB), or the combination of 2 μ M RA plus 60 μ M FAB (RA+FAB) for 4 days. **(B)** Quantification of the percentage of β -tubulin III-positive cells per EB; the results are the mean \pm SE; $n=2$. A statistically significant difference was detected (* $P < 0.05$) between RA+FAB and RA. Scale bar corresponds to 50 μ m.

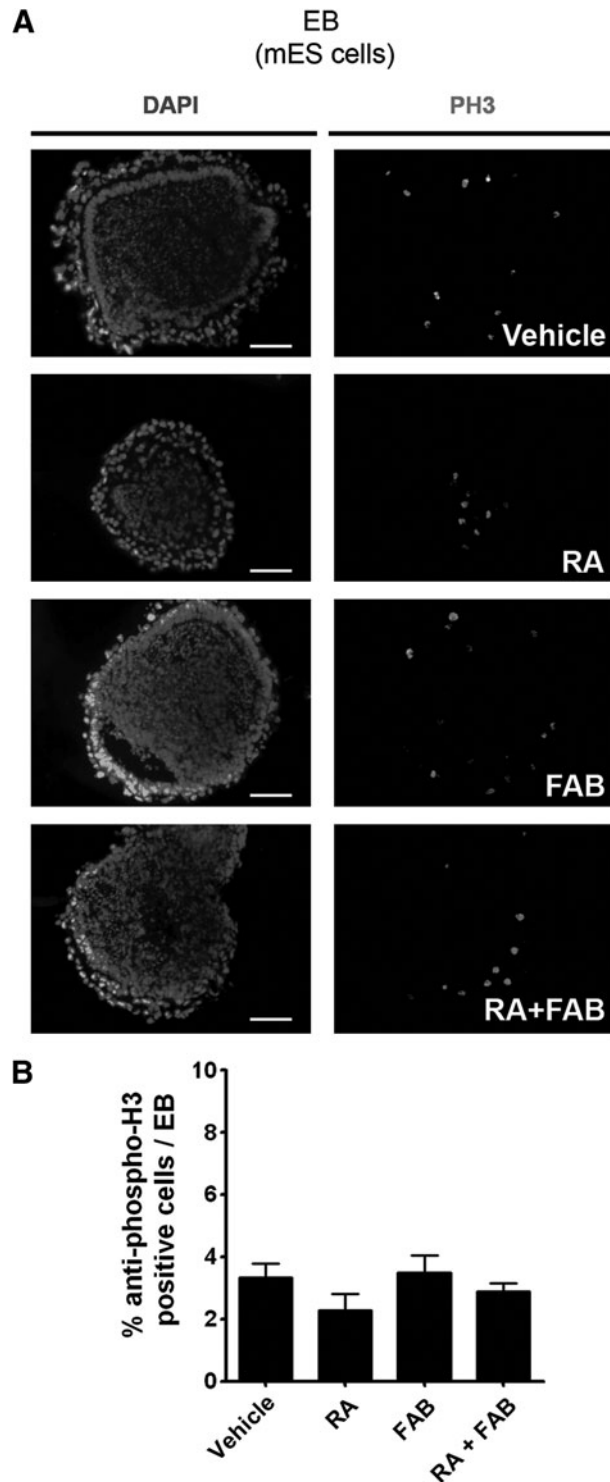


FIG. 5. Agathisflavone does not alter mitosis in EBs. **(A)** Photomicrographs of frozen sections of EBs immunostained with anti-phospho-histone H3 antibodies (PH3); nuclei were counterstained with DAPI. EBs derived from mES cells were incubated with vehicle (0.1% of DMSO) or treated with either 2 μ M RA, 60 μ M agathisflavone (FAB), or the combination of 2 μ M RA plus 60 μ M FAB (RA+FAB) for 4 days. **(B)** Quantification of the percentage of phospho-H3-positive cells per EB by the indicated groups; the results are the mean \pm SE of 3 independent experiments. Scale bar corresponds to 50 μ m.

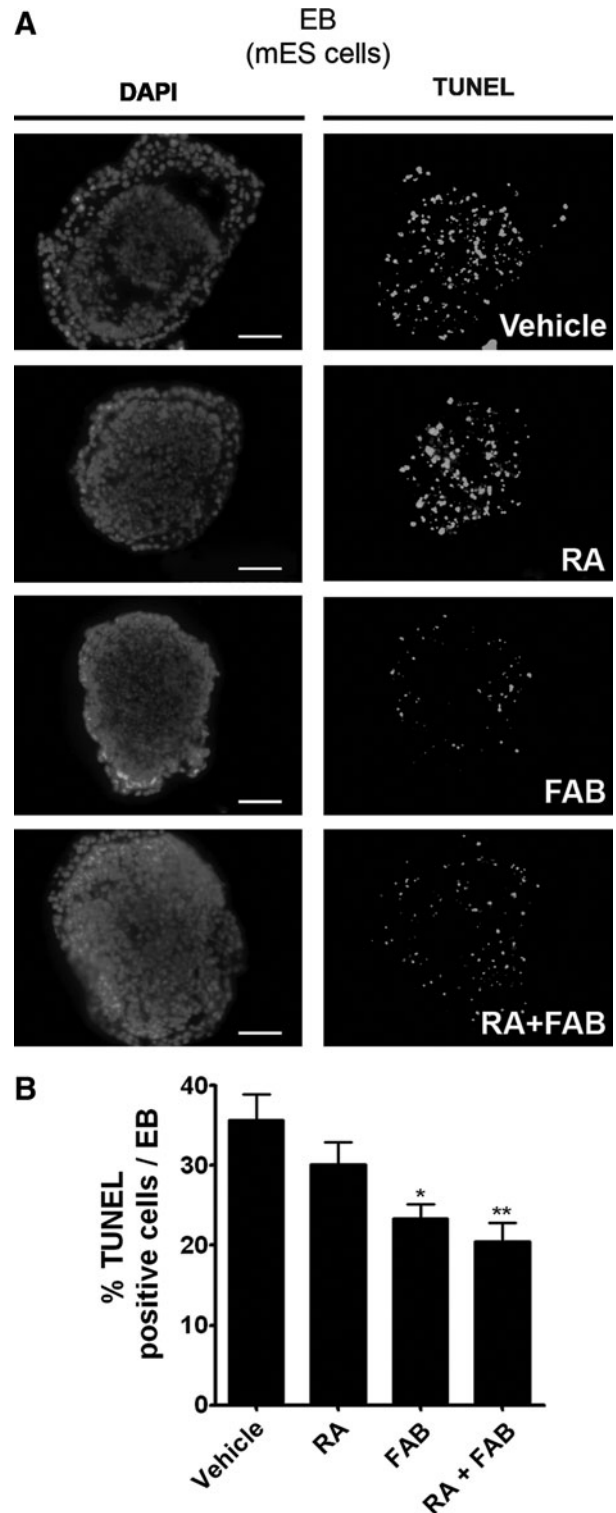


FIG. 6. Agathisflavone increases cell survival in EBs. **(A)** Photomicrographs of frozen sections of EBs after the fluorescent TUNEL assay; nuclei were counterstained with DAPI. EB derived from mES cells were incubated with 0.1% of vehicle (DMSO) or treated with 2 μ M RA, 60 μ M agathisflavone (FAB), or the combination of 2 μ M RA plus 60 μ M FAB (RA+FAB). **(B)** Quantification of the percentage of cell death by the TUNEL assay/total of cells per EB; the values are expressed as the mean \pm SE; $n=3$. * $P<0.05$; ** $P<0.01$ (1-way ANOVA). Scale bar corresponds to 50 μ m.

(Fig. 4). In conclusion, FAB increases the amount of neural progenitor cells induced by RA in both mES and miPS EBs.

Agathisflavone does not affect the mitotic rate, but decreases cell death in EBs

To investigate whether the number of neuronal progenitors was increased by an effect of FAB on proliferation, we evaluated the effects of FAB in mitosis of pluripotent stem

cells growing as EBs. Frozen sections of EBs were processed for immunofluorescence to identify phosphorylated histone H3 (Fig. 5A), a G2/mitosis specific marker [18,19]. The amount of positive cells for phosphorylated histone H3 was similar in the experimental groups (Fig. 5B), suggesting that FAB has no effect on the mitotic rate of both mES and miPS cells.

As some flavonoids, such as casticin, are able to increase the survival of neuronal progenitor cells in vitro [20], we

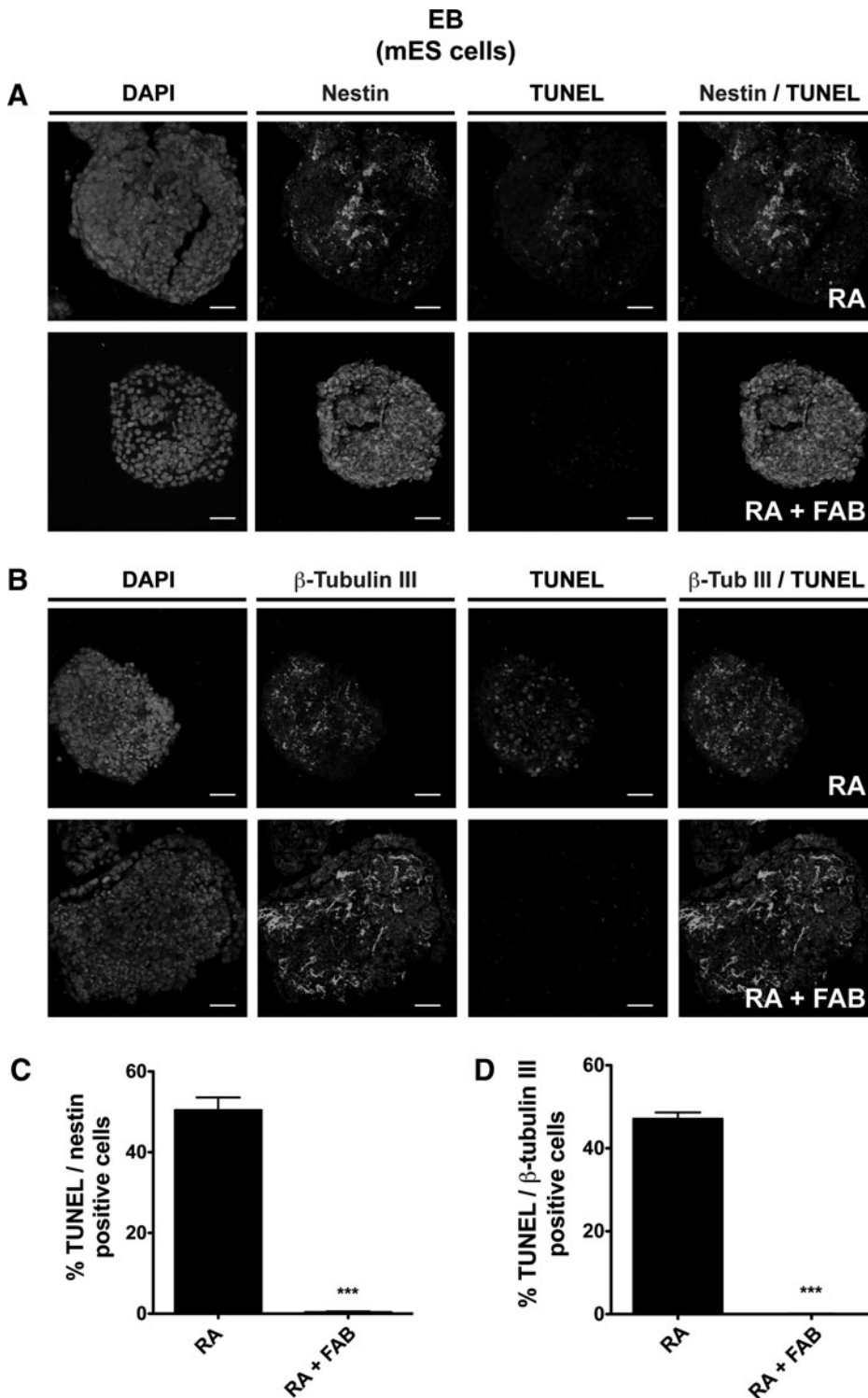


FIG. 7. Agathisflavone promotes the survival of neuroprogenitor cells in EBs. EBs derived from mES cells were treated with 2 μ M RA or the combination of 2 μ M RA plus 60 μ M FAB (RA+FAB). **(A)** Photomicrographs of frozen sections of EBs were immunostained with both anti-nestin and the fluorescent TUNEL assay. **(B)** Photomicrographs of frozen sections of EBs were immunostained with both anti- β -tubulin III and the fluorescent TUNEL assay; nuclei were counterstained with DAPI. **(C)** Histogram showing the percentage of TUNEL-positive nuclei/total of nestin-positive cells per EB. **(D)** Histogram showing the percentage of TUNEL-positive nuclei/total of β -tubulin III cells per EB; the values are expressed as the mean \pm SE; $n=3$. *** $P<0.0001$ (1-way ANOVA). Scale bar corresponds to 50 μ m.

investigated whether FAB could reduce cell death in EBs undergoing neurogenesis. FAB was able to decrease the amount of TUNEL-positive cells in all experimental groups (Fig. 6A, B). Similar antiapoptotic effect of FAB was also observed by immunofluorescence analyses of cleaved caspase-3 within EBs (Supplementary Fig. S3).

To evaluate whether FAB was protecting specifically neuronal progenitor cells, we analyzed EBs with 2-color fluorescent staining for TUNEL and neuronal markers (nestin and β -tubulin III) by confocal microscope (Fig. 7A, B). The percentage of nestin- and β -tubulin III-positive cells undergoing cell death was statistically higher in RA-treated cells (nestin: $50.4\% \pm 3.1\%$; β -tubulin III: $47.1\% \pm 1.6\%$) than within the RA+FAB treated group (both below 1%) (Fig. 7C, D). These results suggested that the effect of enhancing RA-induced neurogenesis is, at least in part, due to FAB promoting the specific survival of neuroprogenitor cells derived from pluripotent stem cells.

Agathisflavone increases the expression of RAR in EBs treated with RA

It is already known that RARs, such as RAR β , are important to neuronal differentiation of mES cells [21] and that sequential activation of both RAR β and RAR α are able to induce neuronal differentiation in cultured neural pro-

genitor cells [22]. In order to characterize a possible mechanism by which FAB enhances neurogenesis induced by RA in pluripotent stem cells, we examined whether FAB increase the expression of RAR in mES after RA treatment. Immunofluorescence assays for RAR α and RAR β in EBs treated with RA alone or with of RA and FAB (Fig. 8A, C) reveal that FAB increases about 3.7 times the expression of both RAR α and RAR β (Fig. 8B, D). These results suggest that FAB enhances neural differentiation induced by RA in mouse pluripotent stem cells by increasing the expression of RAR α and β .

To investigate whether upregulation of RARs were relevant to induce RA-neurogenesis, EBs of mES cells were preconditioned with FAB during EB formation (from d4 to d6; Supplementary Fig. S4). After washing-out, RA incubation was carried out for 4 days (d6 to d10). The expectation was that the preconditioning with FAB would increase the expression of RAR and even after the FAB was washed-out, it would provide an increase on neuronal differentiation induced by RA. The percentage of β -tubulin III-positive cells detected in the group preconditioned with FAB (FAB/RA) was significantly higher ($24.0\% \pm 1.0\%$) than RA treated group ($12.1\% \pm 0.5\%$) or vehicle ($0.3\% \pm 0.1\%$) (Fig. 9), suggesting that preconditioning with FAB increases neuronal differentiation by upregulating RARs even before RA treatment.

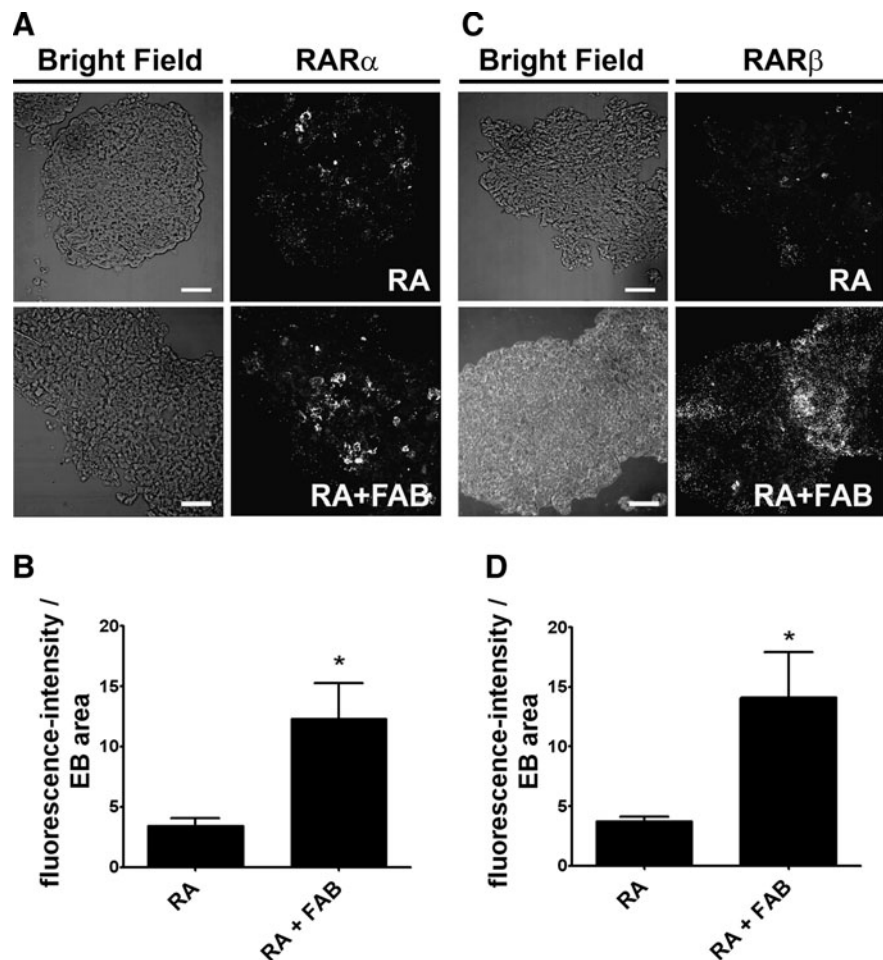


FIG. 8. Agathisflavone increases the expression of RARs α and β in EBs treated with RA. (A, C) Confocal photomicrographs of frozen sections of EBs immunostained (right panels) for RAR α (A) and β (C) and the respective bright field (left panels). EBs cultures, derived from mES cells, were treated with $2\ \mu\text{M}$ RA or the combination of $2\ \mu\text{M}$ RA plus $60\ \mu\text{M}$ agathisflavone (RA+FAB). (B) Quantification of the percentage of RAR α -positive cells per EB by the indicated groups. (D) Quantification of the percentage of RAR β -positive cells per EB by the indicated groups. The results are the mean \pm SE of 3 independent experiments. * $P < 0.05$. RAR, RA receptor.

Discussion

The main feature of pluripotent stem cells is their ability to differentiate into any other cell type. Nevertheless, to design efficient protocols for amplifying differentiated cell populations is still a challenge that demands new strategies and the combination of multiple compounds. In this sense, small molecules have been intensely investigated as inducers of specific differentiation in pluripotent stem cells [23].

Flavonoids are natural polyphenolic compounds present in a variety of vegetables and Chinese herbal medicines [3], which show biological activities including antioxidative [24], anti-inflammatory [25], and antitumoral effects [26–28]. The variety and relatively low toxicity of flavonoids compared with many active plant compounds highlights their potential for therapeutic use. As an example, the flavonoid epicatechin was able to decrease motor neuron specific death in a drug-screening model for amyotrophic lateral sclerosis by reducing reactive oxygen species (ROS) production in mutated astrocytes [29]. Further, polyphenol epigallocatechin gallate and tocotrienol were included as candidates for the treatment of familial dysautonomia disease using familial dysautonomia patient-specific iPSCs [30].

The influence of FAB on proliferation, cell death, and neural differentiation has never been explored in mES and miPS cells. This biflavonoid did not change proliferation or neural differentiation rates in those cells, but it was able to reduce cell death, which was about 35% in 0.1% of DMSO (vehicle) group. The reduction of cell death by FAB is consistent with previous results that show that flavonoids can reduce cell death [10,20]. The exact mechanism by which flavonoids protect against cell death may be vast, as those compounds are widely known not only as antioxidant, but also as activators of PI3 kinase, tyrosine kinase and MAP kinase pathway [4,31,32], enhancers of proteasome activity [33], and modulators of protein assembly and oligomerization [11]. Since DMSO is the solvent most suitable for maintaining the stability of RA and FAB [5,6,9,20,23,28], side effects induced by the vehicle might be an inevitable feature of the protocol. In this context, the association of FAB to the protocol becomes applicable in order to reduce inevitably cell death induced by the DMSO and RA. Nevertheless, the ability to reduce cell death per se opens an opportunity to use flavonoids to increase the survival of differentiated stem cells in vitro. As our data suggest that FAB is promoting the survival of neuroprogenitor cells derived from pluripotent stem cells.

Agathisflavone enhanced the effectiveness of RA as a neural morphogen in both mES and miPS cells, whereas the percentage of neural progenitor cells and young neurons increased more than 12-folds. These observations were associated with an increase in the expression of RAR α and RAR β in ES cells and even the preconditioning of EBs with FAB was able to increase neuronal differentiation once it is presumably upregulating RAR before RA treatment.

Both receptors have shown to be relevant to neurogenesis, since: (i) they are expressed in the subventricular zone of the adult mice [34]; (ii) the sequential activation of RAR β and RAR α induces neuronal differentiation in cultured neural progenitor cells [22]; (iii) RAR β are necessary for RA-induced differentiation, as RAR β mutant in mES cells fail to differentiate, while remained pluripotent [21].

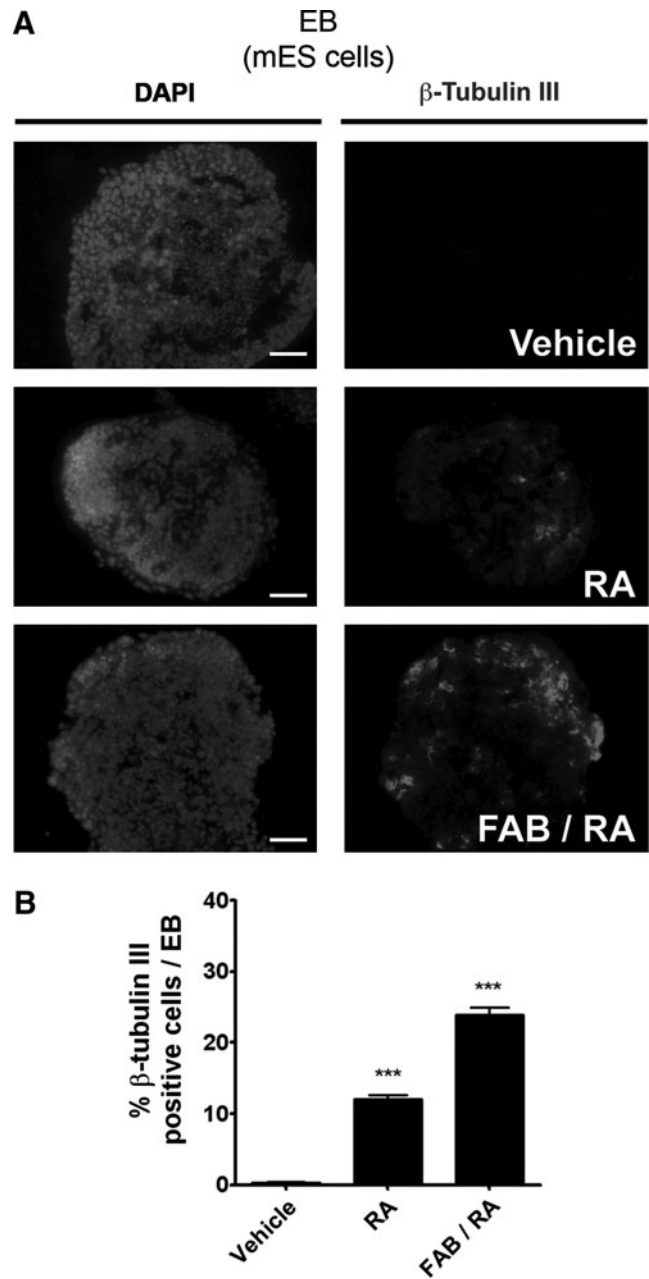


FIG. 9. Pretreatment with agathisflavone increases the number of young neurons differentiated after RA treatment in EBs. **(A)** Photomicrographs of EBs immunostained for anti- β -tubulin III; nuclei were counterstained with DAPI. EBs were incubated with vehicle (0.1% DMSO), RA (2 μ M RA), or pretreated with agathisflavone (60 μ M FAB, d4–d6) followed by washout and 2 μ M RA induction (d6–d10). **(B)** Quantification of the percentage of β -tubulin III-positive cells per EB; the results are the mean \pm SE; $n = 3$. *** $P < 0.0001$. Scale bar corresponds to 50 μ m.

In the present study, we show that agathisflavone (FAB) increases the expression of RAR α and RAR β in ES cells, which probably improves the ability of RA to induce neural differentiation in pluripotent stem cells. It is worth mentioning that flavonoids, such as apigenin, are able to activate estrogen receptors [35], which promote amplification of the RA signaling pathway [36] by increasing the expression of

their receptors. As FAB is an association of 2 apigenins, it is possible that FAB effects as an enhancer of RA-induced neurogenesis is associated to an increase of the amount of RARs in pluripotent stem cells.

Recently, the flavonoid icaritin had been reported to induce neuronal differentiation in mES cells growing as EBs [23]. The authors described that icaritin was able to induce neuronal differentiation by itself when EBs were cultured for at least 10 days in neurobasal medium supplemented with B27. The expression of nestin and β -tubulin III was similar in the icaritin-treated cells and RA alone, suggesting that this flavonoid can act as a substitute of RA rather than an enhancer of RA neurogenic effects.

Taken together, our results indicate that FAB increases cell survival and RA-induced neuronal differentiation in pluripotent stem cells. To our knowledge, this is the first report to describe that naturally occurring biflavonoids regulate apoptosis and neuronal differentiation in both mES and miPS cells. These results may benefit the development of novel protocols to enrich the number of differentiated neurons derived from pluripotent stem cells *in vitro*.

Acknowledgments

This work was supported by Instituto Nacional de Ciência e Tecnologia em Terapia Celular (INCTC); Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ); Conselho Nacional para o Desenvolvimento Científico e Tecnológico (CNPq), Instituto do Milênio de Terapia Gênica, Ministério da Saúde/DECIT, Fundação do Câncer; and Instituto Nacional do Câncer (INCa). The authors thank Ismael Gomes and Severino Galdino for technical assistance.

Author Disclosure Statement

The authors report no conflicts of interest, including personal or financial.

References

- Bain G, WJ Ray, M Yao and DI Gottlieb. (1996). Retinoic acid promotes neural and represses mesodermal gene expression in mouse embryonic stem cells in culture. *Biochem Biophys Res Commun* 223:691–694.
- Sarkar SA and RP Sharma. (2002). All-trans-retinoic acid-mediated modulation of p53 during neural differentiation in murine embryonic stem cells. *Cell Biol Toxicol* 18:243–257.
- Middleton E, C Kandaswami and TC Theoharides. (2000). The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol Rev* 52:673–751.
- Mandel S, O Weinreb, T Amit and MBH Youdim. (2004). Cell signaling pathways in the neuroprotective actions of the green tea polyphenol (-)-epigallocatechin-3-gallate: implications for neurodegenerative diseases. *J Neurochem* 88:1555–1569.
- Mercer LD, BL Kelly, MK Horne and PM Beart. (2005). Dietary polyphenols protect dopamine neurons from oxidative insults and apoptosis: investigations in primary rat mesencephalic cultures. *Biochem Pharmacol* 69:339–345.
- Sagara Y, J Vanhnasy and P Maher. (2004). Induction of PC12 cell differentiation by flavonoids is dependent upon extracellular signal-regulated kinase activation. *J Neurochem* 90:1144–1155.
- Wo Y, D Zhu, Y Yu and Y Lou. (2008). Involvement of NF-kappaB and AP-1 activation in icaritin promoted cardiac differentiation of mouse embryonic stem cells. *Eur J Pharmacol* 586:59–66.
- Wo Y, D Zhu, Y Hu, Z Wang, J Liu and Y Lou. (2008). Reactive oxygen species involved in prenylflavonoids, icaritin and icaritin, initiating cardiac differentiation of mouse embryonic stem cells. *J Cell Biochem* 103:1536–1550.
- Lee MK, SW Lim, H Yang, SH Sung, H Lee, MJ Park, et al. (2006). Osteoblast differentiation stimulating activity of biflavonoids from *Cephalotaxus koreana*. *Bioorg Med Chem Lett* 16:2850–2854.
- Wang CN, CW Chi, YL Lin, CF Chen and YJ Shiao. (2001). The neuroprotective effects of phytoestrogens on amyloid beta protein-induced toxicity are mediated by abrogating the activation of caspase cascade in rat cortical neurons. *J Biol Chem* 276:5287–5295.
- Ehrnhoefer DE, J Bieschke, A Boeddrich, M Herbst, L Masino, R Lurz, et al. (2008). EGCG redirects amyloidogenic polypeptides into unstructured, off-pathway oligomers. *Nat Struct Mol Biol* 15:558–566.
- Mendes CC, MV Bahia, JM David and JP David. (2000). Constituents of *Caesalpinia pyramidalis*. *Fitoterapia* 71:205–207.
- Nones J, TCLS Spohr, DR Furtado, RC Sartore, BS Paulsen, MZP Guimarães, et al. (2010). Cannabinoids modulate cell survival in embryoid bodies. *Cell Biol Int* 34:399–408.
- Marinho PAN, AM Fernandes, JC Cruz, SK Rehen and LR Castilho. (2010). Maintenance of pluripotency in mouse embryonic stem cells cultivated in stirred microcarrier cultures. *Biotechnol Prog* 26:548–555.
- Huangfu D, R Maehr, W Guo, A Eijkelenboom, M Snitow, AE Chen, et al. (2008). Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. *Nat Biotechnol* 26:795–797.
- Marques SA, FM Almeida, AM Fernandes, C Dos Santos Souza, DV Cadilhe, SK Rehen, et al. (2010). Predifferentiated embryonic stem cells promote functional recovery after spinal cord compressive injury. *Brain Res* 1349:115–128.
- Borges HL, C Chao, Y Xu, R Linden and JYJ Wang. (2004). Radiation-induced apoptosis in developing mouse retina exhibits dose-dependent requirement for ATM phosphorylation of p53. *Cell Death Differ* 11:494–502.
- Henzel MJ, Y Wei, MA Mancini, A Van Hooser, T Ranalli, BR Brinkley, et al. (1997). Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma* 106:348–360.
- Kingsbury MA, SK Rehen, JJA Contos, CM Higgins and J Chun. (2003). Non-proliferative effects of lysophosphatidic acid enhance cortical growth and folding. *Nat Neurosci* 6:1292–1299.
- de Sampaio E Spohr TCL, J Stipursky, AC Sasaki, PR Barbosa, V Martins, CF Benjamim, et al. (2010). Effects of the flavonoid casticin from Brazilian *Croton betulaster* in cerebral cortical progenitors *in vitro*: Direct and indirect action through astrocytes. *J Neurosci Res* 88:530–41.
- Chatzi C, CE van den Brink, PT van der Saag, CD McCaig and S Shen. (2010). Expression of a mutant retinoic acid receptor beta alters lineage differentiation in mouse embryonic stem cells. *Stem Cells Dev* 19:951–60.

22. Goncalves MBCV, M Agudo, S Connor, S McMahon, SL Minger, M Maden, et al. (2009). Sequential RARbeta and alpha signalling *in vivo* can induce adult forebrain neural progenitor cells to differentiate into neurons through Shh and FGF signalling pathways. *Dev Biol* 326:305–313.
23. Wang Z, H Wang, J Wu, D Zhu, X Zhang, L Ou, et al. (2009). Enhanced co-expression of beta-tubulin III and choline acetyltransferase in neurons from mouse embryonic stem cells promoted by icaritin in an estrogen receptor-independent manner. *Chem Biol Interact* 179:375–385.
24. Yang CS, J Hong, Z Hou and S Sang. (2004). Green tea polyphenols: antioxidative and prooxidative effects. *J Nutr* 134:3181S.
25. Guardia T, AE Rotelli, AO Juarez and LE Pelzer. (2001). Anti-inflammatory properties of plant flavonoids. Effects of rutin, quercetin and hesperidin on adjuvant arthritis in rat. *Farmaco* 56:683–687.
26. Yang CS, JM Landau, MT Huang and HL Newmark. (2001). Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annu Rev Nutr* 21:381–406.
27. Lambert JD, J Hong, G Yang, J Liao and CS Yang. (2005). Inhibition of carcinogenesis by polyphenols: evidence from laboratory investigations. *Am J Clin Nutr* 81(1 Suppl):284S–291S.
28. Cabrera M, M Simoens, G Falchi, ML Lavaggi, OE Piro, EE Castellano, et al. (2007). Synthetic chalcones, flavanones, and flavones as antitumoral agents: biological evaluation and structure-activity relationships. *Bioorg Med Chem* 15:3356–3367.
29. Marchetto MCN, AR Muotri, Y Mu, AM Smith, GG Cezar and FH Gage. (2008). Non-cell-autonomous effect of human SOD1 G37R astrocytes on motor neurons derived from human embryonic stem cells. *Cell Stem Cell* 3:649–657.
30. Lee G, EP Papapetrou, H Kim, SM Chambers, MJ Tomishima, CA Fasano, et al. (2009). Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs. *Nature* 461:402–406.
31. Schroeter H, C Boyd, JPE Spencer, RJ Williams, E Cadenas and C Rice-Evans. (2002). MAPK signaling in neurodegeneration: influences of flavonoids and of nitric oxide. *Neurobiol Aging* 23:861–880.
32. Balasubramanian S, L Zhu and RL Eckert. (2006). Apigenin inhibition of involucrin gene expression is associated with a specific reduction in phosphorylation of protein kinase Cdelta Tyr311. *J Biol Chem* 281:36162–36172.
33. Maher P. (2008). Proteasome inhibitors prevent oxidative stress-induced nerve cell death by a novel mechanism. *Biochem Pharmacol* 75:1994–2006.
34. Haskell GT and LaMantia A. (2005). Retinoic acid signaling identifies a distinct precursor population in the developing and adult forebrain. *J Neurosci* 25:7636–7647.
35. Mak P, Y Leung, W Tang, C Harwood and S Ho. (2006). Apigenin suppresses cancer cell growth through ERbeta. *Neoplasia* 8:896–904.
36. Prins GS, WY Chang, Y Wang and van Breemen RB. (2002). Retinoic acid receptors and retinoids are up-regulated in the developing and adult rat prostate by neonatal estrogen exposure. *Endocrinology* 143:3628–3640.

Address correspondence to:

Prof. Stevens K. Rehen

Instituto de Ciências Biomédicas

Laboratório Nacional de Células-Tronco Embrionárias

Unidade Rio de Janeiro—LaNCE-RJ

Hospital Universitário Clementino

Fraga Filho—HUCFF

Rua Rodolpho Paulo Rocco

255-4° andar—CEPEDIP

Cidade Universitária—Ilha do Fundão

Rio de Janeiro 21941-913

Brazil

E-mail: srehen@anato.ufrj.br

Received for publication October 04, 2010

Accepted after revision January 30, 2011

Prepublished on Liebert Instant Online January 31, 2011

